Journal of Chromatography, 480 (1989) 129–139
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 957

PERFORMANCE OF AN AUTOMATED INJECTION AND REPLENISHMENT SYSTEM FOR CAPILLARY ELECTROPHORESIS

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SUMMARY

An automated sample introduction system for capillary electrophoresis is described. The sampler allows injection by electromigration and hydrodynamic flow (using a controlled vacuum system). Both methods yielded good linearity of peak area response when the duration of injection or vacuum/voltage level was varied. A novel replenishment system, allowing fresh buffer solution into the capillary and electrolyte vials before each analysis, is shown to improve reproducibility. Test results are shown for a capillary electrophoresis system utilizing 50 μm I.D. capillaries with a pH 7 phosphate buffer.

INTRODUCTION

Capillary electrophoresis (CE) is rapidly gaining acceptance as a high-performance separation technique applicable to a wide range of substances. Recent reviews cover theory, instrumentation and applications of CE[1–4]. After a decade of research, commercial instrumentation for CE is now available. This development should further expand the scope of the technique in terms of its applicability and instrument performance. While the basic designs of CE instruments are similar, instruments vary in the way sample introduction and detection are performed, the degree of automation and in the way data acquisition and data manipulation are done. Compared to high-performance liquid chromatography (HPLC) and gas chromatography (GC) the weak link in the CE instrumentation, in terms of quantitative precision, is the sample introduction process. One should keep in mind though, that it took a number of years before reliable, "splitless" injectors were available in capillary GC. It may be expected that CE will go through a similar development.

Sample introduction in CE has been accomplished in a number of ways. Mikkers *et al.*[5] found that manual injection of sample with a syringe via a septum in an injection block gave undesirable mixing of the sample with the operating buffer. In addition, the accuracy and precision is inadequate when very small amounts of sample are introduced. They also found that with valve injection it was difficult to avoid the detection of undesirable impurities. A sample introduction system with an electric splitting technique designed for 200–300-μm I.D. capillaries was described by Deml *et al.*[6]. Tsuda *et al.*[7] used a rotary-type device for sample injection. Here also, relatively

0021-9673/89/$03.50

large-bore capillaries were used. The small-I.D. capillaries require zero- or low-dead-volume couplings or else unacceptable extra-column band broadening will result. Verheggen *et al.*[8] described a sampling device for CE and capillary isotachophoresis in which the sample solution is introduced into a broadened part of the capillary tube by means of two feeders placed perpendicular to the capillary tube. The device was tested for 250-μm I.D. capillary tubing and yielded good reproducibility.

Sample injection by means of electromigration has been employed by a number of workers[1–4]. In this method, high voltage is applied for a short period of time while the capillary and electrode are positioned in the sample vial. The resulting electroosmotic flow and/or electrophoretic migration drives the sample solution into the capillary. However, the electromigration method is inherently discriminative because the electrophoretic mobilities of individual sample components are different[2]. In a recent paper, Hartwick *et al.*[9] described an "electrosyphon" method designed to prevent sample discrimination.

Other sample introduction methods based on hydrodynamic flow (with syphoning or vacuum suction) have been suggested to prevent sample discrimination. Honda *et al.*[10] obtained good peak area precision with an autosampler in which the sample is raised to a certain height allowing the sample to be syphoned into the capillary. Precision was reported to be dependent on sampling time (amount injected) and tube diameter. Rose and Jorgenson[11] built a similar autosampler which allowed sample introduction by syphoning and electromigration. Area reproducibility was *ca.* 3% relative standard deviation (R.S.D.) for an unretained solute with ten consecutive runs. With a manual sample introduction system, area precision was excessively high, *i.e.* 13.4% R.S.D. Using the electromigration method, the authors found slightly worse precision (4.1% R.S.D.) than with the syphoning method.

In this paper, the design and performance of an automated CE system allowing sample introduction with electromigration as well as with hydrodynamic flow will be described. The goal is to achieve quantitative precision comparable to HPLC. The factors affecting peak area precision and migration time precision in CE will be discussed. Incorporated in the instrument is a "replenishment system" which allows for fresh buffer solution each time an analysis is performed. It will be shown that this replenishment system significantly enhances reproducibility.

EXPERIMENTAL

Instrumentation

A Model M-1200 instrument for CE (Microphoretic Systems, Sunnyvale, CA, U.S.A.) was used in constant voltage mode for all experiments. The instrument was placed in an air-conditioned laboratory. Photodiode array detection was by UV at either 197, 247 or 263 nm. Peak area and migration times calculations were performed with the instrument software. A "PC Integrator" (PE Nelson, Cupertino, CA, U.S.A.) was used for plate count measurements by the statistical moments method. A detailed description of the CE instrument is published elsewhere[12]. The multichannel display mode of this instrument allows for simultaneous monitoring of detector wavelength(s) as well as vacuum, voltage and current signals. Fused-silica capillary tubing, 50 μm I.D. and 375 μm O.D., was purchased from Polymicro Technologies (Phoenix, AZ, U.S.A.). The capillary was cleaned by rinsing with 0.1 *M* NaOH on a day-to-day basis.

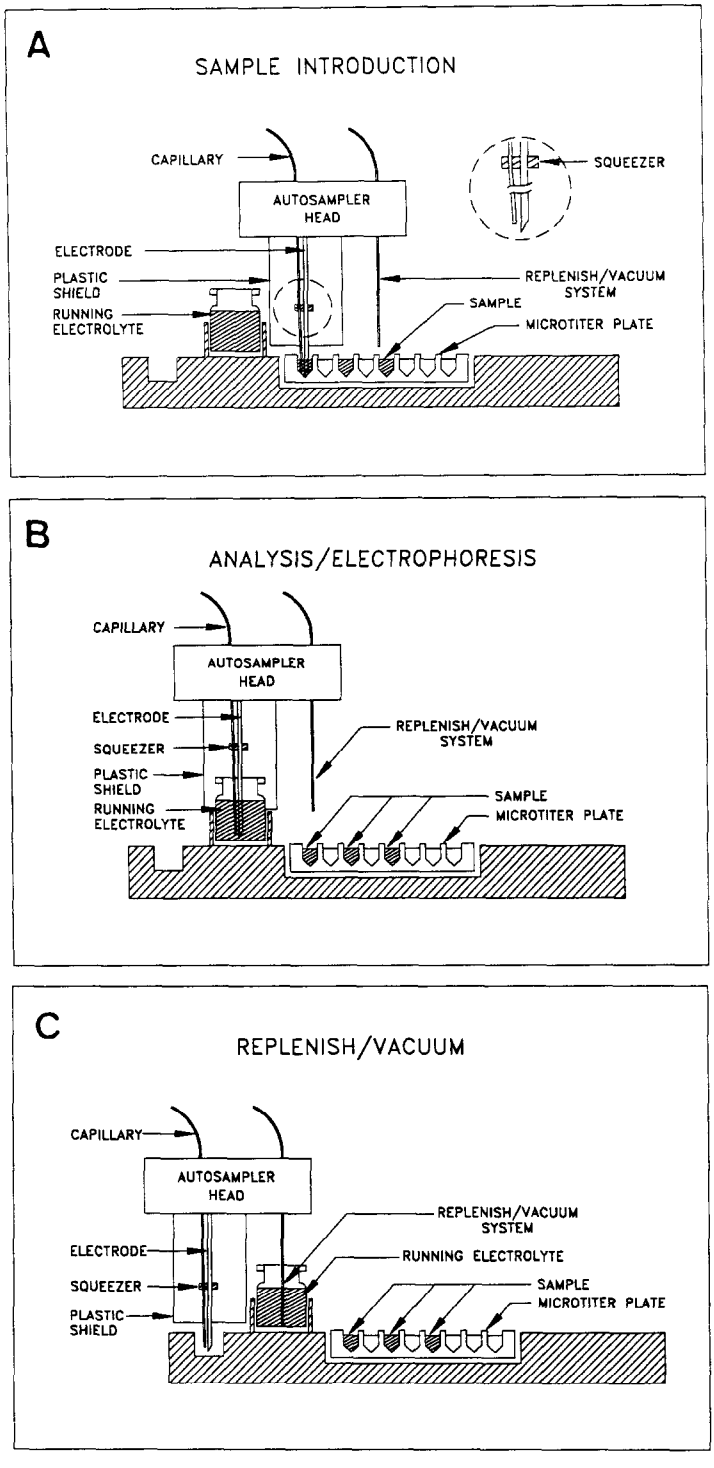

Fig. 1. The autosampler and replenishment system used in the Model 1200 capillary electrophoresis instrument. (A) Sample introduction from one location of the microtiter plate; (B) autosampler position during the electrophoresis run; (C) position of the sampler during running electrolyte replenishment.

Overnight the capillary was stored in distilled water. Dansyl amino acids were purchased from Sigma (St. Louis, MO, U.S.A.). Buffer and sample solutions were filtered prior to electrophoretic analysis. Samples were dissolved in running electrolyte prior to injection.

Autosampler design

A schematic, cutaway drawing of the *x,y,z*-type autosampler is shown in Fig. 1. The high-voltage electrode, capillary and replenishment tube are connected to an autosampler head which can move in any *x*, *y* or *z* direction. In this way, any one of four vials can be used for the "running electrolyte". During CE runs, the autosampler head alternates between a sampling tray and the electrolyte vial. A rubber spacer "squeezer" with two holes ensures that the capillary is held closely to the high-voltage electrode; hence, sampling from small-diameter vials or wells in a microtiter plate can be accomplished. The end of the electrode is beveled-shape to allow piercing a plastic cover sheet (household food wrap) glued to the 96-well microtiter plate (see text). In Fig. 1A, the autosampler is positioned in one of the 96 wells of the microtiter plate to allow sample introduction. A plastic cylinder (radius of 1.5 cm) surrounds the electrode/capillary and is designed to prevent corona discharge. Sample introduction is achieved by instructing the microprocessor to apply a specific vacuum level for a certain time period or, alternatively, by means of applying a voltage for a certain time period. After the sample has been introduced in the capillary, the autosampler head moves to the position shown in Fig. 1B in which the electrode and capillary are positioned in the running electrolyte vials. After the electrophoretic run, the autosampler head moves to the position shown in Fig. 1C. Here the replenishment tube is in the running electrolyte vial. Replenishment of the buffer electrolyte takes place after the original electrolyte solution has been emptied by vacuum into the waste bottle (see *Replenishment system* section).

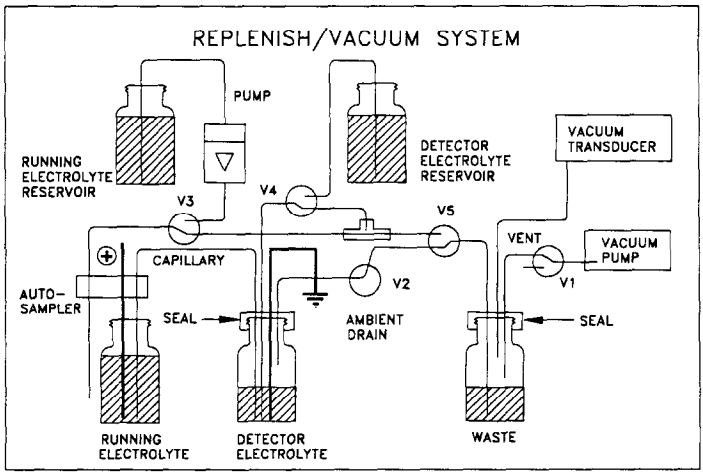

Fig. 2. A schematic of the replenishment and injection systems. Not drawn to scale. See Experimental section for details.

Replenishment system

The valving scheme/plumbing diagram is shown in Fig. 2. The capillary is positioned between a "running" and "detector" electrolyte vial, each with a 2-ml maximum volume. During the refill cycle (valve V3 is in the "on" position), a pump is supplying fresh electrolyte solution to the running electrolyte vial. The vacuum pump, pressure transducer, valve V1, and waste bottle (Fig. 2) serve as a controlled vacuum source. After a specific vacuum level has been reached, the modulator valve (V1) switches the vacuum pump to ambient pressure until the vacuum exceeds the set value. The set vacuum level is maintained by an on/off duty cycle of the modulator valve.

Washing the capillary with electrolyte solution is achieved by drawing a vacuum at the detector electrolyte vial. The electrolytes in both the running and detector electrolyte vials can be sequentially emptied by applying a vacuum with the appropriate valve settings. The refilling of the running electrolyte vial is by means of a pump; the refilling of the detector electrolyte vial takes place by maintaining vacuum and turning on valve V4 for a specified time. When replenishment of both detector and running electrolyte is requested, the following sequence is performed: (1) the running electrolyte is replenished, (2) the capillary is washed, (3) the detector electrolyte is replenished (valve V4 "on") and (4) the electrophoresis is initiated.

RESULTS AND DISCUSSION

In several publications[3,8,10,11,13] factors affecting analytical precision in CE have been mentioned. First, the electrophoretic mobility of a given solute is temperature dependent, increasing at a rate of *ca.* 2%/°C. As the migration time of the solute is inversely proportional to the sum of the electroosmotic flow and solute mobilities[3], changes in electrophoretic mobility will directly affect solute migration times. Therefore, it is important to control the temperature of the environment and/or capillary[14].

Secondly, chemically related factors such as buffer pH, buffer type, and buffer concentration are required for precise control of the electroosmotic flow. This "bulk" flow depends on the ζ potential on the capillary walls. Compared to HPLC, where accurate solvent delivery systems are available to control the flow-rate (and therefore analytical precision), in CE it is more difficult to control the flow of liquid through the capillary. The effect of the electroosmotic flow on the migration time of charged and non-charged species is well documented[1–3,15].Various ways to control and measure the electroosmotic flow have been recently suggested by Wanders *et al.*[16]. McCormick[17] stated that the use of acidic buffers with untreated fused-silica tubing should yield more reproducible separations compared to operation at alkaline pH, as silica becomes soluble at high pH. The author speculated that operation at low pH (*i.e.* at reduced electroosmotic flow) should yield more reproducible separations since, at high pH, changes in ionic strength, pH and capillary wall contamination could result in substantial changes in the magnitude of the electroosmotic flow.

Most laboratories have certain, empirically found procedures for capillary pre-treatment. These may vary according to the type of samples analyzed and the experimental electrophoretic conditions required. Typically, the capillary is periodically cleaned with dilute alkaline solutions[18,19] after which the capillary is flushed with water and running electrolyte. In between each sample run, most workers wash

the capillary with running electrolyte. With respect to analytical precision, the effect of electrolyte reservoir contamination after an electrophoretic run is less documented. Nielsen *et al.*[20] found it necessary to replace the buffer solutions in the reservoir every four to six runs. The automated replenishment system in our current system is designed to start each run with fresh electrolyte solutions in the reservoirs (see Experimental section).

Thirdly, because of the inherently small dimensions of the electrophoretic separation medium, only nanoliters of sample are introduced in the capillary. This places strong demands on the design of injection systems with respect to system performance (*i.e.* to prevent excessive band broadening). Also, while the possibility of sampling from sample sites as small as a few miroliters often is pointed out as an advantage of CE, it poses a problem in terms of quantitation as evaporation and/or contamination from small sampling sites can occur. In our system, sampling can take place from a 96-well microtiter plate. Sample evaporation is minimized by glueing (using a photo-mounting spray) household food wrap over the microtiter plate; during sample introduction the capillary and electrode puncture the plastic (see Experimental section). In addition, sample contamination can occur when injection by electromigration is carried out. Electrochemical byproducts are formed as a result from the temporary flow of current through the sample solution.

From the above discussion it is clear that it may not be trivial to obtain reproducibility standards similar to state-of-the-art HPLC instruments as perhaps more chemistry-related factors are involved. However, as we will show below, by using an automated CE system, this goal may be realized.

The need for an automated replenishment system became clear after experiments with the electromigration injection method failed to give acceptable peak area and migration time precision. The results obtained with and without the replenishment system are summarized in Table I. A 50 cm × 50 μm capillary was used with a 25 m*M* phosphate buffer, pH 7.0. The solute injected was uracil which under these conditions migrates with the speed of the electroosmotic flow. Nine consecutive runs were carried

TABLE I

EFFECT OF THE REPLENISHMENT SYSTEM ON PRECISION USING ELECTROMIGRATION AND HYDRODYNAMIC INJECTION

Data shown are from nine consecutive runs using uracil as the test substance, 0.1 mg/ml. Capillary 50 cm × 50 μm I.D., 25 m*M* phosphate buffer at pH 7.0. Voltage during the electrophoresis was 25 kV.

Replenishment	*Injection time (s)*	*Injection voltage/vacuum*	*Migration, R.S.D. (%)*	*Area, R.S.D. (%)*
Electromigration injection				
Yes	1	10 kV	0.8	4.8
No	3	5 kV	2.4	16.4
Yes	3	5 kV	0.5	4.2
Hydrodynamic injection				
Yes	1	0.5 bar	2.6	5.3
No	1	0.5 bar	2.5	6.9
Yes	3	0.5 bar	1.4	2.4

out at a constant voltage of 25 kV. The amounts injected were calculated to be 0.1–1.0 ng for 5–10-kV injection voltages during 1–3 s. The replenishment cycle included a 5-min wash of the capillary with running electrolyte. The results of Table I reveal a 2.4 and 16.4% R.S.D. for migration times and peak areas, respectively, when no replenishment was used. With replenishment, precision was much better, *i.e.* 0.5 and 4.2% R.S.D., respectively. The R.S.D. values are dependent on the duration of injection (see also Table II). With a 1-s injection at 10 kV, the precision was slightly worse: 0.8 and 4.8% R.S.D., respectively.

It should be noted that with hydrodynamic injection (bottom half of Table I), no significant differences in precision were observed between sets of runs with or without replenishment in between analyses. During the electromigration injection, electrochemical byproducts are formed which eventually contaminate the sample well; hence, the electroosmotic flow pertinant to the sample injection changes slightly and therefore the amount injected. As with the electromigration method, injections with longer duration (3 *vs.* 1 s) yield better results.

The effect of the replenishment system is further illustrated with a multicomponent test mixture of dansyl amino acids in Fig. 3. The same phosphate buffer as in the experiment of Table I was used. With no replenishment in between the runs, the last eluting compound, dansyl aspartic acid, is no longer visible in the bottom electropherogram which represents run 10. With replenishment, precision with respect to peak areas and migration times is quite good, as the results in Table II indicate. Again, better precision (especially with peak areas) is obtained when the vacuum

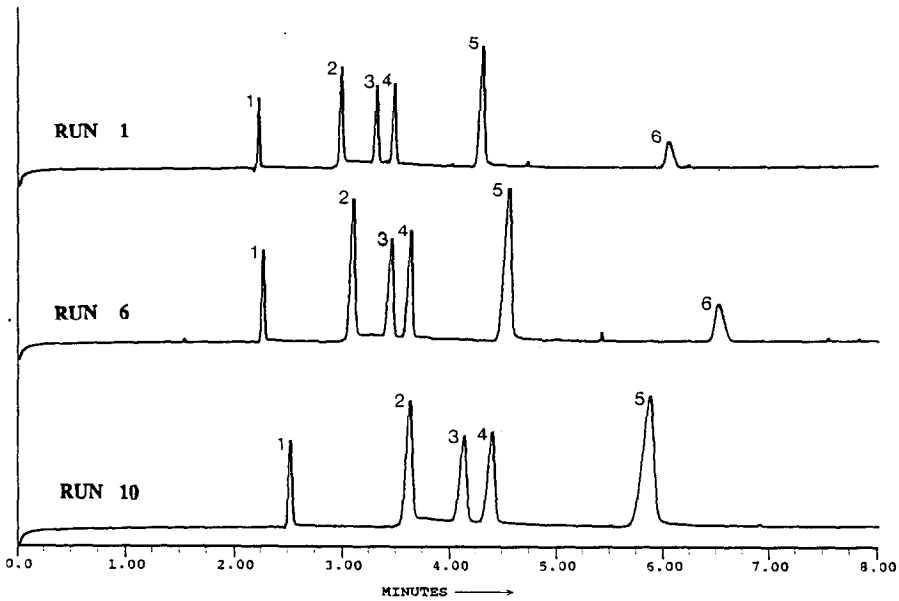

Fig. 3. Three selected runs (1, 6 and 10) from a set of ten which show the effect of not using buffer replenishment on migration times. A test mixture of dansyl amino acids, at $5 \cdot 10^{-4}$ *M* each were used. Peaks: 1 = lysine; 2 = didansyl lysine; 3 = isoleucine; 4 = alanine; 5 = didansyl cystine; 6 = aspartic acid. Conditions: the buffer was 25 m*M* phosphate, pH 7.0, 50 cm × 50 μm I.D. capillary and voltage of 20 kV. Detection: UV, 247 nm.

TABLE II

PRECISION WITH A MULTICOMPONENT TEST MIXTURE USING THE REPLENISHMENT SYSTEM BETWEEN EACH OF THE NINE CONSECUTIVE RUNS

A test mixture of dansyl amino acids, identified in Fig. 3, at $5 \cdot 10^{-4}$ *M* each were used. Capillary 50 cm × 50 μm I.D., buffer 25 m*M* phosphate at pH 7.0, 20 kV.

	R.S.D. (%) of migration time for peak					*R.S.D. (%) of area for peak*				
	1	*2*	*3*	*4*	*5*	*1*	*2*	*3*	*4*	*5*
Hydrodynamic, 1 s at 0.2 bar	0.6	0.8	0.9	1.0	1.2	8.3	10.3	7.7	7.7	7.4
Hydrodynamic, 3 s at 0.2 bar	0.5	0.6	0.7	0.7	0.9	4.1	4.0	4.3	4.3	5.3
Electromigration, 1 s at 5 kV	0.6	0.7	0.7	0.7	0.9	2.7	2.2	2.8	2.2	2.4

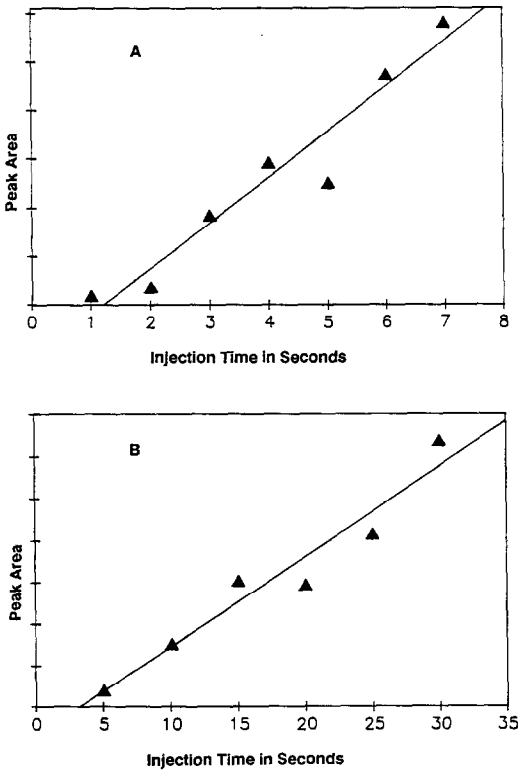

Fig. 4. Effect of changing the time of injection on the peak area. (A) Hydrodynamic injection; (B) electromigration injection.

injection is carried out for a longer time. This is because the frequency of the on/off duty cycle of the modulator valve (valve V1 in Fig. 2) is not fast enough at small injection times; at larger injection times the differences in vacuum level are averaged out. Experiments with much longer injection times and smaller vacuum levels are currently under investigation. With the dansyl amino acids, the best precision was obtained with the electromigration method, *i.e.* 0.6–0.9% R.S.D. for migration times and 2.2–2.8% R.S.D. for peak areas. The results of Tables I and II are clearly superior compared to precision obtained with post-column densitometric scanning of electrophoresis gels and comparable to recently published data with automated CE instrumentation[10,11,13].

It should be noted that the results of Tables I and II represent CE conditions in which the electroosmotic flow is present. Quantitative precision may improve under conditions of minimal electroosmotic flow (*e.g.* with coated capillaries or at low pH conditions). Finally, precision is probably worse under conditions of higher current levels or where the buffer capacity is inadequate.

Linearity of peak area response

Both injection methods were evaluated by a set of experiments in which the time of injection was varied while the vacuum level/voltage was kept constant (Fig. 4A and

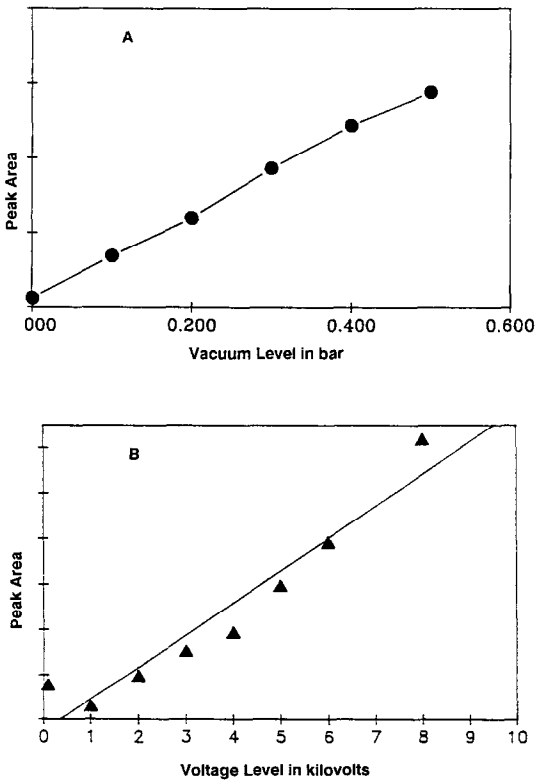

Fig. 5. Effect of changing the vacuum/voltage level of injection on the peak area at a constant injection time. (A) Hydrodynamic injection; (B) electromigration injection.

B). In another set of experiments, shown in Fig. 5A and B, the vacuum level/voltage was varied while the time of injection was kept constant. The test solute injected was uracil at a concentration of 0.1 mg/ml. Electrophoretic runs were performed at 25 kV. Under these conditions the amount of material injected was calculated to be between 0.1 and 1 ng. The preliminary results of Fig. 5 show good linearity in the range shown, indicating that both methods of injection can be utilized with 50 μm I.D. capillaries. However, as will be discussed next, the range of injection is limited by sample overload and inadvertant injection.

The effect of sample introduction on peak efficiency and resolution has been studied by various researchers (see ref. 21 and references cited theirein). Instrument and/or chemistry (*e.g.* adsorption[19] and focusing effects[5]) related factors affect the peak efficiency which can be obtained. In practice, peak efficiency is often traded for selectivity, speed, or convenience. Rose and Jorgenson[11] did not find a significant difference in peak efficiency comparing the electromigration to the hydrodynamic injection method. Apparently, other sources of band broadening obscured a possible difference. In accordance with the findings of Grushka and McCormick[21], an inadvertent injection is produced when the capillary just touches the sample solution even though no vacuum is applied and care is taken to prevent syphoning of sample into the capillary. This is illustrated in Fig. 6 using uracil as the test solute. It has been suggested[21] that density differences between the sample and the buffer may be responsible for this phenomenon which affects the overall separation efficiency.

Fig. 6 also shows the effect of overloading on peak shape and plate number. The inadvertent injection at zero vacuum level yields the highest plate number (N), *i.e.* 187 600. Higher vacuum levels produced peaks with lower efficiency and even distorted peak shape. Experiments using electromigration injection (not shown), also show similar results of overloading on peak shape and efficiency. Therefore, the perfor-

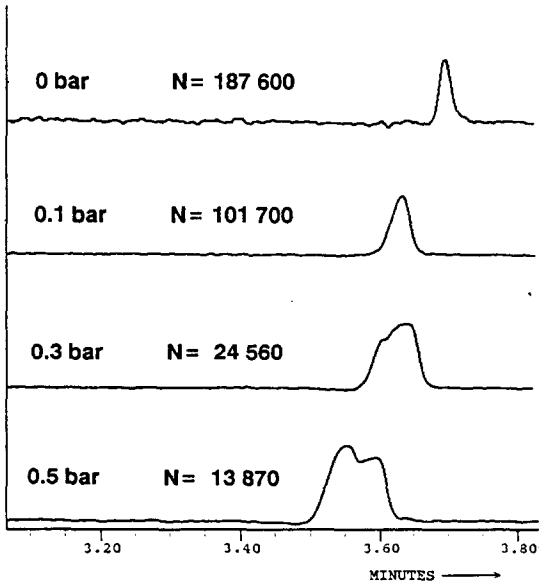

Fig. 6. Effect of sample overload on peak shape and efficiency using hydrodynamic injection. Sample and test conditions as in Table I. The top trace shows the inadvertent injection.

mance of the sample injection system in CE is bounded on the low end by the inadvertent injection (high efficiency but poor detection limits) and on the high end by degraded separation efficiency (but improved sample detection limits). Although extremely high plate numbers in excess over a million plates have been reported in CE under certain conditions[16], plate numbers in the 100 000–200 000 range give perhaps a more realistic and practical representation of current CE separation efficiencies[3].

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